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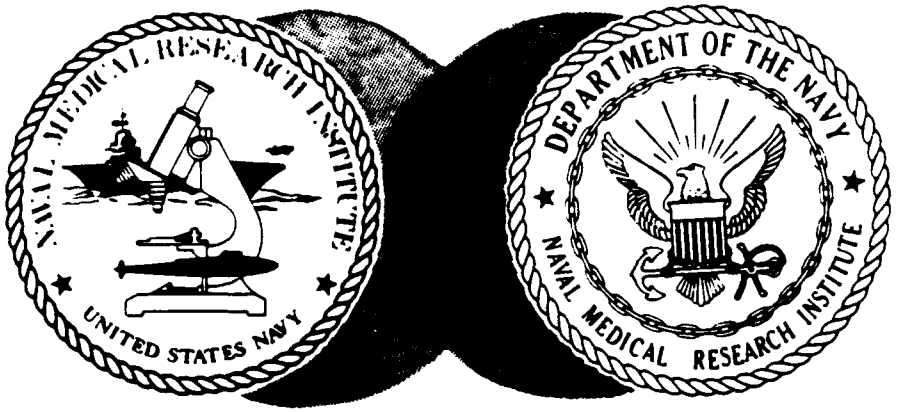

80-98

BIOLOGIC EFFECTS OF MICROWAVE EXPOSURE. II. STUDIES ON THE MECHANISMS CONTROLLING SUSCEPTIBILITY TO MICROWAVE-INDUCED INCREASES IN COMPLEMENT RECEPTOR-POSITIVE SPLEEN CELLS

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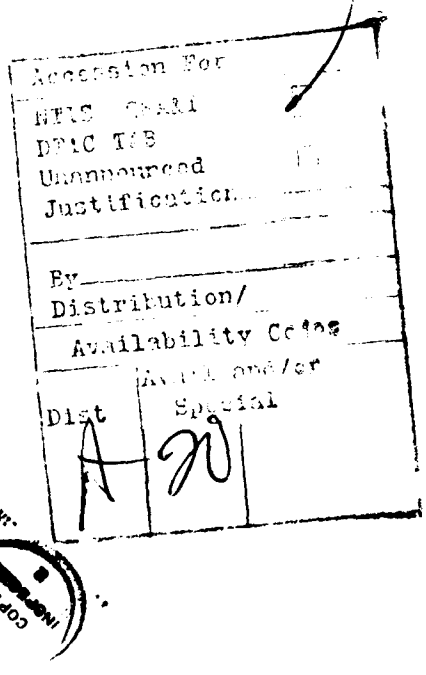
20. In attempting to evaluate the mechanisms responsible for susceptibility to the inductive increase in splenic complement receptor-positive (CR^+) cells following exposure to 2450-MHz microwaves, it was found that sensitivity to microwave-induced CR^+cell increases was under genetic control. In particular, evidence was accumulated suggesting that regulation was under the control of a gene or genes closely associated with but outside of the mouse major histocompatibility complex (H-2). All responsive strains of mice tested were of the $H\text{-}2^k$ haplotype, while mice of the $H\text{-}2^a$, $H\text{-}2^b$, $H\text{-}2^d$ and $H\text{-}1i^s$ haplotypes were refractory to the microwave-induced increases in CR^+ cells. By utilizing certain $H\text{-}2^k$ strains of mice that were genetically unable to respond to endotoxin, we were able to show that these strains of mice responded to microwaves, but not to endotoxin, by increasing CR^+ cells. Microwave-induced increases in CR^+cells were not mimicked by the intraperitoneal injection of hydrocortisone. Athymic mice responded to microwave exposure, indicating that this event was not regulated by the T-cell population. Mice less than eight weeks old were found not to be susceptible to exposure to 2450-MHz microwaves. These studies indicate that microwaves do induce changes in the population of cells with specific cell-surface receptors, that susceptibility to these changes is under genetic control, and that it is unlikely that endotoxin, corticosteroids, or regulatory T cells play a significant role in the mechanisms regulating these increases.



Bioelectromagnetics 1:405–414 (1980)

Biologic Effects of Microwave Exposure. II. Studies on the Mechanisms Controlling Susceptibility to Microwave-Induced Increases in Complement Receptor-Positive Spleen Cells

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In attempting to evaluate the mechanisms responsible for susceptibility to the inductive increase in splenic complement receptor-positive (CR^+) cells following exposure to 2450-MHz microwaves, it was found that sensitivity to microwave-induced CR^+cell increases was under genetic control. In particular, evidence was accumulated suggesting that regulation was under the control of a gene or genes closely associated with but outside of the mouse major histocompatibility complex (H-2). All responsive strains of mice tested were of the $H\text{-}2^k$ haplotype, while mice of the $H\text{-}2^a$, $H\text{-}2^b$, $H\text{-}2^d$ and $H\text{-}1i^5$ haplotypes were refractory to the microwave-induced increases in CR^+ cells. By utilizing certain $H\text{-}2^k$ strains of mice that were genetically unable to respond to endotoxin, we were able to show that these strains of mice responded to microwaves, but not to endotoxin, by increasing CR^+ cells. Microwave-induced increases in CR^+cells were not mimicked by the intraperitoneal injection of hydrocortisone. Athymic mice responded to microwave exposure, indicating that this event was not regulated by the T-cell population. Mice less than eight weeks old were found not to be susceptible to exposure to 2450-MHz microwaves. These studies indicate that microwaves do induce changes in the population of cells with specific cell-surface receptors, that susceptibility to these changes is under genetic control, and that it is unlikely that endotoxin, corticosteroids, or regulatory T cells play a significant role in the mechanisms regulating these increases.

Key words: 2450-MHz radiation, complement receptors, endotoxin, T cells, genetic control

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INTRODUCTION

We have previously reported that 30-minute exposures of mice to 2450-MHz microwaves (0.6 W forward power, 12–15 mW/g) in an environmentally controlled waveguide facility induced marked increases in complement receptor-positive (CR^+) and Fc receptor-positive (FcR^+) spleen cells [Wiktor-Jedrzejczak et al, 1977a, b]. Studies of functional alterations in the immune system have shown that exposure to 2450-MHz microwave radiation induces weak stimulatory effects on splenic B cells, as measured by the increased responsiveness of these cells to B-cell mitogens and the increase of spontaneous IgM-secreting, antibody-forming spleen cells [Wiktor-Jedrzejczak et al, 1977c]. These functional perturbations were not associated with a concomitant increase in cell proliferation in the spleen, bone marrow, or peripheral blood or with an increase in body temperature [Wiktor-Jedrzejczak et al, 1980], nor were T cell-mediated immune responses altered.

Analysis of lymphocyte surface membrane markers has provided conclusive evidence that the CR^+ lymphocytes affected by 2450-MHz radiation were B cells, ie, they were positive for surface immunoglobulin (sIg^+) and negative for Thy-1 [Sulek et al, 1980]. These results indicate that microwaves, under these exposure conditions, stimulate the maturation of a subpopulation of B lymphocytes in the spleens of exposed mice.

While the kinetics and threshold conditions of the induced increase in splenic CR^+ cells have been established firmly and the affected cell population identified as B cells [Sulek et al, 1980], we have very little understanding of the basic biological mechanisms involved in the interaction of microwaves and B lymphocytes. During the course of our initial studies, mice of various ages and from several inbred strains were exposed to 2450-MHz radiation to allow us to select the most susceptible age and strain. We found that certain strains seemed to be refractory to this inductive event, whereas others were susceptible. The finding of responder-nonresponder strains indicates that the event being studied is under the control of a small number of genes. The availability of genetically mutant strains of mice has allowed researchers to evaluate the role of various regulatory agents and events in many biological systems. We therefore decided to study the mechanisms controlling the microwave-induced increases in CR^+ cells using genetically characterized inbred strains of mice.

MATERIALS AND METHODS

Mice

CBA/J, C3H/HeJ, CE/J, B10.BR, AKR/J, B10.D2, BALB/c, DBA/2, A/J, C57BL/6, B10.A(5R) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. CBA/N and CBA/H T6J nu/nu mice were obtained from the Small Animal Section, National Institutes of Health, Bethesda, Maryland.

Microwave Exposure

Exposure conditions and methods of microwave dosimetry were as previously described [Wiktor-Jedrzejczak et al, 1977a]. Mice were exposed for 30 minutes to 2450-MHz, continuous-wave microwaves at a forward power of 0.6 W in an environmentally controlled waveguide facility [Ho et al, 1973] at the Bureau of Radiological Health, Rockville, Maryland. The mice were then transported back to the Naval Medical Research Institute, Bethesda, Maryland, where the immunological tests were performed. A Hewlett-Packard (Palo Alto, Calif.) 8616A signal generator with a 491C amplifier tuned to 2450

MHz ± 500 Hz and using a tuneable coherent synchronizer, model 251 (Sage Laboratories, Natick, Mass.), was used as the microwave source. Mice from each group were exposed individually to 2450-MHz microwaves while being restrained in a plastic (polystyrene) holder that was previosuly found not to absorb or reflect a significant amount of microwave radiation. During exposure, a constant temperature of 24 ± 0.5 °C, a relative humidity of 50 ± 5%, and a constant airflow of 6.4 m/min were maintained inside the waveguide The average specific absorption rate (SAR), in mW/g, was calculated from measurements of forward, reflected, and transmitted power and the weight of the mouse [Youmans and Ho, 1975]. Six microwave-exposed mice and six sham-exposed mice composed each experimental group.

Evaluation of Spleen Cells for Complement Receptor

The mice were killed by cervical dislocation, their spleens removed, and single-cell suspensions prepared in RPMI-1640 medium (Microbiological Associates, Walkersville, Md), containing 10% fetal calf serum (FCS) and 1% Hepes buffer (medium-10% FCS). Erythrocytes were lysed by hypotonic shock with AKC lysing buffer (Microbiological Associates). After washing with medium-10% FCS, the percentage of CR^+ spleen cells was determined by the method of Bianco et al [1970]. In this assay system, a lymphocyte with three or more adherent erythrocytes was considered as a positive rosette. Two hundred lymphocytes were always counted in each sample, and the percentage of CR^+ cells was determined. The increase in CR^+ spleen cells was calculated using the formula:

$$\frac{\%\,CR^+ \text{ cells in microwave-exposed} - \%\,CR^+ \text{ cells in sham-exposed}}{\%\,CR^+ \text{ cells in sham-exposed}} \times 100$$

The erythrocyte-antibody-complement (EAC) reagent was made of sheep red blood cells (SRBC) and a 19S fraction of human anti-SRBC antibody; fresh mouse serum was used as a source of complement. The EAC reagent also contained 0.01M ethylenediaminetetraacetic acid (EDTA) to prevent binding of EAC complexes to granulocytes or macrophages.

Previous studies [Wiktor-Jedrzejczak et al, 1977a] indicated that the increase in CR^+ cells was first seen on the third day after exposure; the number of cells peaked on day 6, and was reduced by day 9. Therefore, in the current study, assays for CR^+ spleen lymphocytes were performed three and six days following microwave or sham exposure.

The unpaired Student's *t*-test was performed on either the raw data or on arc sin-transformed data to determine the statistical significance of the induced increase in CR^+ spleen cells.

Treatment of Mice With Lipopolysaccharide (LPS) and Hydrocortisone

Six mice per group were injected intraperitoneally (i.p.) with Escherichia coli 0111:B4 LPS (Difco, Detroit, Mich.) (0.1 or 1.0 μg) or hydrocortisone (HC) sodium succinate (Solu-cortef, Upjohn Co., Kalamazoo, Mich.) (0.4 or 4.0 mg). The level of CR^+ spleen cells was assayed on days 3, 6, and 9 for mice injected with LPS and on days 3 and 6 for those receiving HC.

RESULTS

Effect of Age on Susceptibility to the Inductive Effects of Microwaves

The levels of CR^+ spleen cells were measured in CBA/J mice of various ages three and six days following a 30-minute exposure to 2450-MHz microwaves as described. Mice six weeks of age and younger were refractory to the inductive effects of 2450-MHz microwaves

(Table 1). Mice younger than eight weeks of age normally have low levels of CR^+ cells. Statistically significant increases in splenic CR^+ cells were not detected until the mice were 12 weeks old. The induced response peaked on day 3 in 30-week-old mice, suggesting that perhaps the older mice possessed a larger subpopulation of cells that were susceptible to the inductive effects of microwaves.

Genetic Susceptibility to the Inductive Effects of Microwaves

The ontogeny of CR^+ spleen cells has been shown to be under the dual control of at least two genes, one of which is closely linked to the H-2 region [Gelfand et al, 1974a, b]. Therefore, we investigated the possibility that susceptibility to the inductive effects of microwaves was also associated with the H-2 haplotype. Various genetically defined strains of mice (age 12 weeks) were exposed to 2450-MHz microwaves as previously described. It was determined (Table 2) that most mice bearing the $H\text{-}2^k$ haplotype showed marked increases in splenic CR^+ cells, whereas mice bearing the $H\text{-}2^a$, $H\text{-}2^b$ and $H\text{-}2^d$ haplotypes exhibited little or no increase.

Failure of Endotoxin to Induce CR^+ Increases in Mutant Strains

From the data in Table 2, we observed that two strains of mice (CBA/N and C3H/HeJ) known to possess genetic defects that alter their responsiveness to LPS [Scher et al, 1977; Watson and Riblet, 1974] were susceptible to the inductive effects of microwaves. To test the hypothesis that the inductive increase in splenic CR^+ might be due to in situ release of endotoxin, we injected 12-week-old CBA/J, C57BL/6, CBA/N, and C3H/HeJ mice with 0.1 μg or 1.0 μg LPS i.p. and monitored the change in CR^+ spleen cells on days 3 and 6. There was no correlation between the susceptibility to the inductive effects of 2450-MHz microwave radiation and the ability to respond to LPS (Table 3). CBA/J mice responded to both LPS and 2450-MHz radiation with an increase in splenic CR^+ cells, C57BL/6 mice responded only to LPS, while CBA/N and C3H/HeJ mice responded to the microwave radiation but not to LPS. However, while both doses of LPS induced an increase in CR^+ CBA/J spleen cells that displayed identical kinetics to 2450-MHz microwaves, HC induced a marked depression of CR^+ CBA/J spleen cells on day 3 that returned to normal levels by day 6 (Fig. 1). These data collectively suggest that endotoxin and corticosteroids are unlikely to be involved in the inductive process.

Role of Regulatory T Cells in the Microwave-Induced Increase in CR^+ Cells

The use of the congenitally athymic nude mouse (nu/nu) has allowed the study of the role of thymus-derived (T) cells in many immune responses [Kindred, 1978]. Since this strain of mouse lacks mature T cells, any biologic events that occur naturally or can be induced in this mouse can be assumed to be outside the regulation of T cells. CBA/H T6J nu/nu mice responded as well as normal CBA/J mice (Table 2) following exposure to 2450-MHz microwaves. From this we conclude that this event is not regulated by the T-cell population.

DISCUSSION

These studies used genetically characterized strains of mice to evaluate the mechanisms involved in the inductive increase in CR^+ spleen cells following a single 30-minute, near-field exposure to 2450-MHz microwaves.

TABLE 1. Levels of CR^+ Spleen Cells in CBA/J Mice of Various Ages Following a 30-Min Exposure to 2450-MHz Radiation

	% CR^+ Spleen Cells[a]					
	Day 3			Day 6		
Age (weeks)	SAR (mW/g)	Sham-exposed	Microwave-exposed	SAR (mW/g)	Sham-exposed	Microwave-exposed
3	10.34	15.0 ± 1.9	15.9 ± 2.2	10.5	18.7 ± 1.4	16.8 ± 1.6
4	9.3	18.8 ± 1.1	20.8 ± 2.1	9.8	18.6 ± 1.6	22.4 ± 2.2
6	14.6	25.1 ± 26.8	26.8 ± 2.4	13.3	25.9 ± 0.5	30.2 ± 0.8
12	12	24.2 ± 1.1	31.5 ± 1.3*	12.3	22.2 ± 1.2	34.1 ± 1.6**
30	10.3	24.3 ± 2.4	38.4 ± 2.2**	10.1	24.4 ± 4.6	29.1 ± 5.8

[a]Levels are listed ± SE.
*$P < 0.01$.
**$P < 0.001$.

TABLE 2. Increase in CR^+ Spleen Cells in Various Strains of Mice After a 30-Min Exposure to 2450-MHz Radiation

	% CR^+ Spleen Cells[a]					
	Day 3			Day 6		
Strain (haplotype)	SAR (mW/g)	Sham-exposed	Microwave-exposed	SAR (mW/g)	Sham-exposed	Microwave-exposed
CBA/J (k)	15.1	24.2 ± 1.1	31.5 ± 1.3*	13.3	22.2 ± 1.2	34.1 ± 1.6**
CBA/J nu/nu (k)	19.3	28.5 ± 1.0	37.0 ± 1.3*	–	–	–
CBA/N (k)	13.7	15.5 ± 1.4	22.0 ± 1.7*	11.4	14.8 ± 1.3	23.7 ± 1.7**
C3H/HeJ (k)	13.4	20.2 ± 0.7	23.4 ± 0.8*	13.0	21.3 ± 2.0	27.9 ± 1.6*
AKR/J (k)	14.0	15.8 ± 1.9	25.1 ± 1.7*	12.1	26.4 ± 1.8	34.9 ± 1.5**
CE/J (k)	12.2	19.6 ± 1.9	24.2 ± 2.3*	15.4	36.5 ± 2.5	35.2 ± 1.0
C58/J (k)	14.9	31.8 ± 1.7	31.4 ± 1.8	16.2	30.2 ± 1.5	28.7 ± 1.7
B10.BR (k)	17.8	23.5 ± 2.6	23.6 ± 1.2	18.6	23.5 ± 2.6	27.1 ± 2.9
B10.D2 (d)	14.7	26.0 ± 1.5	28.0 ± 2.1	16.1	18.2 ± 1.3	19.5 ± 0.9
DBA/2 (d)	13.3	25.2 ± 1.7	27.4 ± 2.0	13.3	23.9 ± 1.1	31.5 ± 2.3*
BALB/c (d)	15.7	14.6 ± 0.9	15.2 ± 1.4	12.3	14.6 ± 0.9	15.4 ± 0.7
A/J (a)	13.0	20.0 ± 2.3	14.8 ± 0.7	14.8	24.4 ± 1.4	29.3 ± 2.7
CBA/N × DBA/2 (k/d)	14.9	14.3 ± 0.2	20.8 ± 0.4**	14.4	22.0 ± 0.6	20.5 ± 2.0
C57BL/6 (b)	12.5	25.3 ± 1.0	26.5 ± 0.8	17.4	25.6 ± 1.3	29.6 ± 2.1
B10.A(5R) (i5)	14.7	32.8 ± 0.7	29.3 ± 1.6	–	–	–

[a]Levels are listed ± SE.
*$P < 0.01$.
**$P < 0.001$.

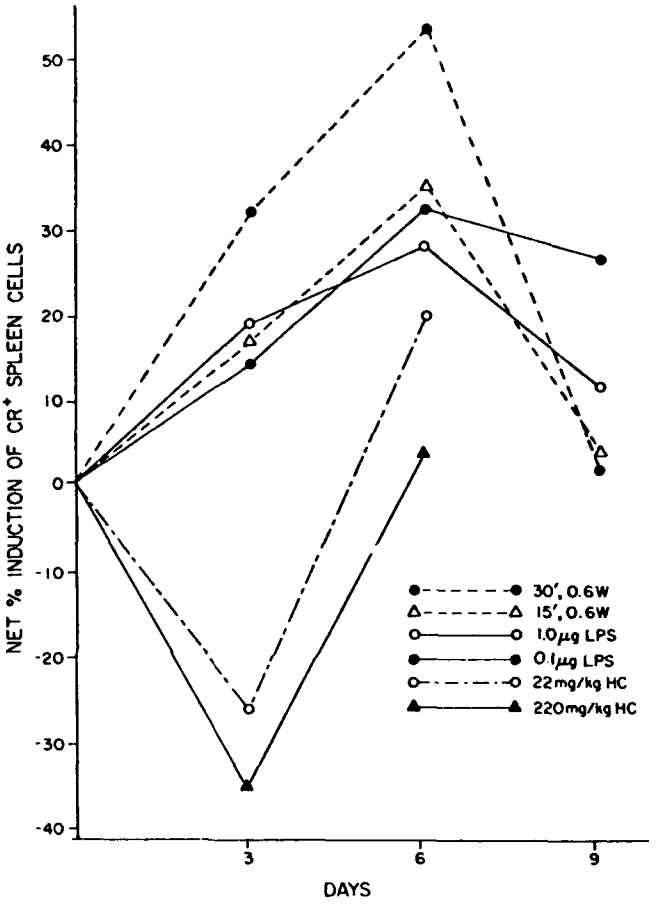

Fig. 1. Kinetics of the net changes in CR^+ CBA/J spleen cells following either exposure to 2450-MHz microwaves (0.6 W) for 15 or 30 minutes (15' or 30') or i.p. injections of LPS or HC. Calculated on the basis of dose/body weight, the 0.4 mg dose of HC was the equivalent of 22 mg/kg and the 4.0 mg dose of HC the equivalent of 22 mg/kg. Six mice were included in each group. An indication of the variability of response for microwave-exposed mice and for LPS-treated mice can be obtained from Table 3.

In previous papers [Wiktor-Jedrzejczak et al, 1977a, c, 1980; and Sulek et al, 1980], we presented evidence that exposure to 2450-MHz microwaves induced the maturation of CR^- to CR^+ B cells. Gelfand et al [1974a] showed that CR^+ spleen cells were infrequent in animals less than two weeks of age, and adult levels of such cells develop gradually over the first six weeks of life. The question we asked was "Can exposure to 2450-MHz microwaves accelerate the rate of maturation of CR^- to CR^+ cells in young mice so that CR^+ levels are the same as adults?" Data presented in Table 1 indicates that mice six weeks old and younger were refractory to the inductive effects of microwaves. If microwaves were inducing the maturation of CR^- to CR^+ cells, then these cells first undergo in situ maturation before becoming susceptible to the effects of 2450-MHz microwaves. The requirement

of in situ maturation was further indicated by the observation that 30-week-old mice exhibited higher responses on day 3 than day 6. This indicated to us that the older mice possessed a larger population of B cells susceptible to the inductive effects of microwaves.

An important finding was that susceptibility to the inductive effects of microwaves appeared to be under genetic control. To our knowledge, this is the first report of a functional alteration in the immune system induced by exposure to microwaves that is under genetic control. The data in Table 2 indicates that only mice of the H-2^k haplotype responded to exposure to 2450-MHz microwaves with an increase in CR^+ spleen cells. The nature of this genetic regulation is unknown. Genes controlling immune responses to individual antigens (Ir genes), mixed lymphocyte responses (MLR genes), serum complement level (Ss-Slp), and the cell-surface major histocompatibility antigens, (glycoproteins K and D [Klein et al, 1978]) are all located within the H-2 complex. The I region within the H-2 complex is known to code for immune-associated (Ia) glycoprotein antigens that are expressed on the surface of mature T and B cells and are involved in enhancing or suppressing B-cell immune responses [Vadas et al, 1976; Murphy et al, 1977; Okuda et al, 1977; Tada et al, 1978]. The susceptibility of CBA/H T6J nu/nu mice to microwave radiation indicates that the induced increase in CR^+ cells is not regulated by the T-cell population.

At this time, we are unable to determine whether the genes controlling susceptibility to microwaves are located near or far from the H-2 complex. Failure of the B10.BR mouse (Table 2) to respond suggests that the essential genes are outside the classical H-2 gene segment, since this animal is coisogenic: it has H-2^k haplotype, but all of the other genes are of the background strain C57BL/10, which does not respond to microwave radiation. However, more congenic strains need to be tested. The absence of the essential genes in CE/J and C58/J mice could explain why these H-2^k strains failed to exhibit an increase in CR^+ cells following microwave exposure.

Ontogenetic studies have revealed that the expression of CR^+ is preceded by the expression of sIg and Ia antigens in at least two independent differentiation steps [Hämmerling et al, 1976]. While each of these sequential stages of maturation and differentiation can be mediated by LPS [Hämmerling et al, 1975], data in this study (Table 3) provides evidence that LPS is not the sole agent that can provide this stimulus. A significant increase in CR^+ cells in mice unresponsive to LPS can be induced by microwave exposure.

In our studies, we made the arbitrary requirement that a lymphocyte must bind at least three erythrocytes to be considered CR^+ [Bianco et al, 1970]; therefore, any B cell binding only one or two erythrocytes is considered CR^- even though these cells may in fact possess low densities of surface CR. The absorption of microwave energy could be inducing an increase in the density of CR on the surface of weakly CR^+ B cells via a still-unknown mechanism that occurs at the cellular level. Conclusive proof of this theory may reside in experiments currently being performed to quantify the density of CR on the surface of B cells from microwave-exposed mice.

Although we have been unsuccessful in identifying the exact mechanisms involved in the induction of an increase of splenic CR^+ B cells following exposure to 2450-MHz radiation, we have shown that corticosteroids and endotoxin are not involved in the regulatory mechanisms and that the event is not dependent upon mature T cells. The inductive process is mediated in genetically susceptible strains of mice by a product of a gene or genes located outside of the H-2 complex. In a previous paper [Sulek et al, 1980], we have shown that the increase in CR^+ spleen cells was not a thermogenic response to microwave exposure. We cannot rule out the possibility that macrophages may play a role in the inductive events.

TABLE 3. Ability of LPS to Mimic Microwave-Induced Increases in CR^+ Spleen Cells on Day 6

		% CR^+ Spleen Cells[a]							
		CBA/J		CBA/N		C3H/HeJ		C57BL/6	
Treatment	Dose	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
2450 MHz[b]	15 min	22.7 ± 1.4	30.7 ± 1.2*	–	–	–	–	–	–
	30 min	22.2 ± 1.2	34.1 ± 1.6*	14.8 ± 1.3*	23.7 ± 2.0	23.7 ± 1.7*	27.9 ± 1.6**	25.6 ± 1.3	29.6 ± 2.1[c]
LPS	0.1 μg	23.0 ± 3.3	30.8 ± 3.8**	12.3 ± 1.6	10.5 ± 1.3[c]	28.6 ± 2.5	21.7 ± 2.2[c]	15.0 ± 1.4	26.8 ± 1.9*
	1.0 μg	25.8 ± 4.0	33.1 ± 1.6**	10.8 ± 2.0	12.8 ± 0.8[c]	28.6 ± 2.5	24.3 ± 2.0[c]	15.0 ± 1.4	23.8 ± 1.9*

[a] Levels are listed ± SE.
[b] 0.6 W forward power.
[c] Not significant.
*$P < 0.001$.
**$P < 0.01$.

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